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1 **Association between hypoxic volume and underlying hypoxia-induced gene**
2 **expression in oropharyngeal squamous cell carcinoma (OPSCC)**

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28
29 **Running Title (50 chars):** Hypoxia biomarkers from ⁶⁴Cu-ATSM PET/CT imaging

30 **ABSTRACT**

31 **Background:** Hypoxia imaging is a promising tool for targeted therapy but the links between
32 imaging features and underlying molecular characteristics of the tumour have not been
33 investigated. The aim of this study was to compare hypoxia biomarkers and gene expression
34 in oropharyngeal squamous cell carcinoma (OPSCC) diagnostic biopsies with hypoxia
35 imaged with ^{64}Cu -ATSM PET/CT.

36 **Methods:** ^{64}Cu -ATSM imaging, molecular and clinical data were obtained for 15 patients.
37 Primary tumour SUV_{max} , tumour to muscle ratio (TMR) and hypoxic volume were tested for
38 association with reported hypoxia gene signatures in diagnostic biopsies. A putative gene
39 signature for hypoxia in OPSCCs (hypoxic volume-associated gene signature, HVS) was
40 derived.

41 **Results:** Hypoxic volume was significantly associated with a reported hypoxia gene
42 signature ($\rho=0.57$, $P=0.045$), but SUV_{max} and TMR were not. Immunohistochemical
43 staining with the hypoxia marker carbonic anhydrase 9 (CA9) was associated with a gene
44 expression hypoxia response ($\rho=0.63$, $P=0.01$). 16 genes were positively and 5 genes
45 negatively associated with hypoxic volume (adjusted $P<0.1$; eight genes had adjusted
46 $P<0.05$; HVS). This signature was associated with inferior 3-year progression-free survival
47 ($\text{HR}=1.5$ [1.0-2.2], $P=0.047$) in an independent patient cohort.

48 **Conclusion:** ^{64}Cu -ATSM defined hypoxic volume was associated with underlying hypoxia
49 gene expression response. A 21-gene signature derived from hypoxic volume from patients
50 with OPSCCs in our study may be linked to progression-free survival.

51

52 **Keywords (3 to 12):** Head and neck cancers; oropharyngeal carcinoma; PET/CT imaging;
53 hypoxia; RNA-sequencing.

54

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58

59 **ABBREVIATIONS**

60 FFPE: formalin-fixed, paraffin-embedded

61 HVS: hypoxic volume-associated gene signature

62 OPSCC: Oropharyngeal squamous cell carcinoma

63 SUV: Standardised uptake value

64 TMR: Tumour to muscle ratio

65

66

67

68 INTRODUCTION

69

70 Hypoxia has been extensively investigated in head and neck squamous cell carcinoma
71 (HNSCC) and the association with poor outcome is well known (Nordsmark *et al*, 2005). A
72 number of therapeutic interventions can target global or focal hypoxia in tumours
73 (Hendrickson *et al*, 2011; Rischin *et al*, 2010), however the selection of patients with hypoxic
74 tumours who would benefit from these interventions has been the major limiting factor for
75 translation into clinical practice.

76 Accurate detection and quantification of hypoxic tumours is essential to identify
77 patients who have aggressive, treatment resistant disease. Various methods have been
78 investigated but with inconsistent and sometimes conflicting results, lacking the sensitivity
79 and specificity needed for clinical utility (Aebersold *et al*, 2001; Overgaard *et al*, 2005).
80 Hypoxic gene expression signatures from biopsy samples could have predictive value
81 (Toustrup *et al*, 2011; Winter *et al*, 2007) but are not able to quantify hypoxia or provide
82 information on its the spatial distribution within tumours. Combining hypoxic gene signatures
83 and ⁶⁴Cu-ATSM PET imaging biomarkers may enable a more comprehensive assessment of
84 the hypoxic status of tumours.

85 ⁶⁴Cu-ATSM is a PET radiotracer that has been shown to accumulate in hypoxic and
86 other conditions of mitochondrial dysfunction (Donnelly *et al*, 2012; Lewis *et al*, 1999).
87 Although the exact mechanism has not been elucidated and uptake may be dependent on
88 cell or tumour type, clinical studies have clearly demonstrated the potential as an imaging
89 biomarker that warrants further investigation, especially in HNSCC, with clear advantages
90 over the nitroimidazole-based hypoxia tracers (Grassi *et al*, 2014; Minagawa *et al*, 2011;
91 Sato *et al*, 2014).

92 Our study hypothesis was that hypoxia gene signatures from diagnostic FFPE biopsy
93 samples would be associated with uptake of ⁶⁴Cu-ATSM in patients with OPSCC, and a
94 signature could be developed to identify patients with OPSCC who would benefit from further

95 investigation with hypoxia imaging. This would, in turn, provide more information on the level
96 and distribution of hypoxic regions, which could potentially guide treatment.

97 MATERIALS AND METHODS

98

99 Patients

100 Fifteen patients with newly diagnosed histologically proven stage III-IV squamous cell
101 carcinoma of the oropharynx to be treated with standard radical concomitant chemoradiation
102 were prospectively recruited as part of an initial pilot phase of the study. HPV status was
103 determined by p16 IHC and by in-situ hybridisation for high risk subtype DNA. Patients
104 received a static ^{64}Cu -ATSM PET/CT scan of the head and neck approximately 1 week prior
105 to the start of their treatment. Time between biopsy and imaging was recorded (Table 1,
106 Supplementary Figure S7). On inspection of clinical follow-up there was one reported death
107 (not disease-specific) and all other patients so far have no recurrence (median time-to-follow
108 up, 24 months). Research Ethics Committee approval was obtained for the study (reference
109 12/LO/1123) and all patients gave written and voluntary consent.

110

111 Image acquisition and analysis

112 Detailed methods for image acquisition and analysis are provided as Supplementary
113 Information. In summary, patients were injected with 545 ± 27 (range 486 - 577) MBq of
114 ^{64}Cu -ATSM followed by an uptake period of 60 minutes. The first patient acquisition was
115 performed on a GE Discovery VCT PET/CT scanner (General Electric Medical Systems,
116 Waukesha, Wisconsin, USA). All subsequent patients were imaged on the GE Discovery
117 710 PET/CT scanner. The ^{64}Cu -ATSM PET/CT images were interpreted by a nuclear
118 medicine physician and clinical oncologist using Hermes Hybrid Viewer version 2.2C
119 (Hermes Medical Solutions, Stockholm, Sweden). PET uptake was assessed semi-
120 quantitatively using standardised uptake values (SUV) normalised to patient body weight
121 determined using the following equation:

122
$$\text{SUV}_{\text{bw}} = \frac{\text{^{64}Cu-ATSM activity concentration measured in the tumour (Bq/cc)} \times$$

123
$$(\text{patient body weight (kg)/injected activity of ^{64}\text{Cu-ATSM (Bq) decay corrected to the}$$

124
$$\text{time of injection}) \times 1000 \text{ g/cc}}{\text{patient body weight (kg)}}$$

Visible lesions on PET with uptake higher than background muscle uptake were considered hypoxic. Background uptake was evaluated by placing fixed 2.5 cm spheres over bilateral posterior neck muscles on the CT images to guide correct positioning, copying the spheres onto the PET images and calculating the average SUV_{mean} . Regions of interest (ROIs) were outlined in multiple planes using a set zoom, SUV scaling and colour scale. An initial region was drawn using an automatic segmentation seeded region growing tool on each scan slice and manually edited. The seeded region growing tool starts with a seed pixel within the tumour and then adds pixels to the region in all direction. The operator determined the extent of pixel expansion and final region outlined. ROIs were then summed to create a volume, or the hypoxic volume. For each primary tumour SUV_{max} , tumour to muscle ratio (TMR), tumour SUV_{mean} and hypoxic volume were measured. TMR was determined by the ratio of tumour SUV_{max} to the average SUV_{mean} of the posterior neck muscles.

Immunohistochemistry, RNA extraction, sequencing and analysis

Diagnostic biopsy slides were reviewed by a head and neck pathologist to confirm the diagnosis, and the tumour outline marked on the slide without further selection. Sections from FFPE tumour blocks were placed onto slides for immunohistochemical staining for carbonic anhydrase 9 (CA9), an endogenous marker for hypoxia, as previously described (Watson *et al*, 2003). Blocks were then scored with a surgical blade to correspond with the tumour regions on the slides. Ten micrometre thick sections of the tumour area were cut on a microtome (minimum 10 sections, minimum tumour area 100 mm²) and sent to BGI (BGI TechSolutions, China). BGI performed RNA extraction, RNA-sequencing (seq) and small RNA-seq (Illumina HiSeq 2000), filtered the reads to remove adaptors and low quality reads. RNA-seq reads aligned to reference genome (hg19; BWA v0.7.10-r789) were obtained from BGI and read counts per gene were enumerated using htseq-count (HTSeq v0.6.1p1 (Anders *et al*, 2015); union of exons). Small RNA reads were obtained from BGI and aligned to microRNA (miRNA) mature sequences (MirBase v21 (Kozomara & Griffiths-Jones, 2011) using bowtie2 v2.2.5 (Langmead & Salzberg, 2012) with local alignment (--local -a). Read

counts per miRNA were enumerated using reads which were uniquely mapped among reported alignments. Differential expression analyses from count data, and read count normalisation (rlog-transform) for visualisation and clustering, were performed using DESeq2 v1.6.3 (Love *et al*, 2014). Differential expression analysis of HPV status was performed using DESeq2 with HPV status as the co-variable. Genes associated with hypoxic volume were identified using DESeq2 with hypoxic volume as a continuous co-variable. Gene set enrichment and leading edge analysis (GSEA v2.2.2 (Subramanian *et al*, 2005); pre-ranked tool; minimum set size=5; 1000 permutations) was performed on genes pre-ranked by fold-change (per unit increase of hypoxic volume) and filtered for coverage (baseMean > 200). Gene sets with names containing the phrase 'hypoxia', 'HIF1' or 'HIF2' were pre-selected from all MSigDB curated gene sets (c2.all.v5.1.symbols.gmt (Subramanian *et al*, 2005)). Gene signature scores were estimated from normalised (rlog-transformed) read counts using a weighted sum of Z-scores for each gene in the respective gene list, with weights (+1,-1) according to the direction of expression in the original gene signature.

Analysis of external gene expression data sets

The following series were retrieved from the Gene Expression Omnibus (an international public genomics data repository): GSE686 (Chung *et al*, 2004) and GSE65858 (Wichmann *et al*, 2015). Follow-up information about progression-free survival (GSE65858) was obtained from GEO, and follow-up information about recurrence-free survival (GSE65858) was obtained from the original publication (Chung *et al*, 2004). A set of pre-defined exploratory analyses of follow-up were performed. Kaplan-Meier plots were used to inspect the hypoxic volume signature score by upper quartile, interquartile and lower quartile of the score values, samples in the upper quartile versus all other samples, and samples in the lower quartile versus all other samples. After inspection of the Kaplan-Meier plots, exploratory log-rank tests were performed for three year follow-up and for the whole follow-up period, and hazard ratios were fitted to the same time periods using univariable Cox proportional hazards models.

181

182

183 **Statistical analysis**

184 Mann-Whitney U tests, Kaplan-Meier plots, log-rank tests and Cox proportional hazard
185 model fits were performed in the R environment, v3.1.2 and 'survival' package v2.38 (R Core
186 Team, 2014). Exact Wilcoxon-Mann-Whitney tests were performed using the 'coin' package
187 v1.1-2. Cox models p-values report the Wald test. Unless otherwise stated, two-tailed tests
188 are reported. $P < 0.05$ was considered to be statistically significant.

RESULTS

Patient characteristics are summarised in Table 1. There were no immediate or late adverse reactions after tracer injection. Uptake was detected in all 15 primary tumours on ^{64}Cu -ATSM PET scans (Figure 1A). Two patients had diagnostic tonsillectomies at diagnosis but uptake was seen in residual disease. The mean SUV_{max} for all 15 patients was 3.32 ± 0.85 (range 2.12–4.85) and the mean TMR was 4.51 ± 1.82 (range 1.84–9.00) (Table 1). SUV_{max} and TMR were not significantly different between HPV-positive and HPV-negative patients. Tonsillectomy patients were excluded from analyses using hypoxic volume and SUV_{mean} .

The mean hypoxic volume in 13 primary tumours was $12.54 \text{ cm}^3 \pm 11.53$ (range 0.68–36.89) and the mean SUV_{mean} was 2.34 ± 0.45 (range 1.71–3.11) (Table 1). Supplementary Figure S1 demonstrates examples of hypoxic volume delineation. Increased uptake was detected in 22 lymph nodes on ^{64}Cu -ATSM PET scans out of 31 abnormal lymph nodes reported on CT imaging. Lymph node uptake appeared visually to be more heterogeneous than uptake in the primary tumour, especially in large nodes, which showed uptake in the periphery with central regions of no uptake, likely secondary to necrosis (Supplementary Figure S2). In general the uptake in the nodes was lower than the primary. The mean SUV_{max} of the nodes was 2.43 ± 0.93 (range 1.39–5.80) and the mean TMR 3.29 ± 2.41 (range 1.35–13.18) (Supplementary Table S1).

The differential gene expression between HPV-positive and HPV-negative samples were compared with previously published signatures (Keck *et al*, 2015; Pyeon *et al*, 2007) as an initial screen to assess the RNA sequencing from FFPE samples (Supplementary Figure S3). Overall the pattern of gene expression profiles in our HPV-positive versus HPV-negative samples were readily distinguishable and consistent with previous studies. There was no difference in hypoxic volume, TMR or SUV_{max} between HPV-positive and HPV-negative samples.

217

218 To establish whether RNA-seq data from the diagnostic biosies could report gene
219 expression responses to hypoxia, we initially compared the gene expression of our samples
220 with two different published hypoxia-associated signatures which have been used to assess
221 hypoxia: a signature comprising genes regulated by hypoxia in head and neck cancers
222 (Winter *et al*, 2007) and the 15-gene hypoxia classifier (Sorensen *et al*, 2015). There was
223 high correlation between the two different signatures in our samples (Supplementary Figure
224 S4C). Hypoxic volume was significantly associated with both the 15-gene hypoxia classifier
225 ($P=0.045$; Spearman's $\rho=0.57$, two-tailed test, Figure 1D) and the Winter *et al.* gene list
226 ($P=0.05$; Spearman's $\rho=0.56$, two-tailed test). SUV_{max} and TMR were not significantly
227 associated with these hypoxia signatures; on inspection, four patient samples exhibited low
228 scores for the 15-gene hypoxia signature but had among the highest values of SUV_{max} and
229 TMR (Figure 1D). There were no significant differences in baseline characteristics between
230 this 'subgroup' and the other samples, and these patients were amongst the oldest in the
231 study (Mann-Whitney U, $W=3$, $P=0.02$).

232

233 To verify that hypoxic imaging volume and RNAseq-derived hypoxia gene expression
234 response were associated with hypoxia in patient tumours, CA9 protein
235 immunohistochemistry (IHC), a marker for hypoxia, was performed on biopsy samples.
236 Among the image features, hypoxic volume showed a positive trend with CA9 IHC scores
237 (Mann-Whitney U one-tailed, threshold median of hypoxic volume, $P=0.06$ (Figures 1B and
238 S4A); Spearman's $\rho=0.43$, $P=0.15$ (Supplementary Figure S4A)). SUV_{max} and TMR were
239 not associated with CA9 (Figures 1B and S4A). Furthermore, the 15-gene hypoxia classifier
240 score was significantly associated with CA9 IHC scores (Spearman's $\rho=0.63$, $P=0.01$
241 (Supplementary Figure S4B)). Genes ranked according to association with hypoxic volume
242 were found to be enriched for previously curated hypoxia-associated gene sets with
243 expression response to hypoxia or downstream of HIF-1 (Supplementary Table S2,
244 Supplementary Figure S5).

245

246 The 15-gene hypoxia classifier (Sorensen *et al*, 2015) was originally derived and
247 tested in multiple cancer types and may represent hypoxia gene expression responses that
248 are commonly found across differing tumour types (Sorensen *et al*, 2015; Winter *et al*, 2007).
249 We hypothesised that alternative gene signatures could accurately evaluate the hypoxic
250 phenotypes in OPSCCs. We used the ⁶⁴Cu-ATSM hypoxic volume together with mRNA
251 expression from biopsy samples to identify a gene signature for hypoxic imaging phenotypes
252 in OPSCCs. Hypoxic volume was significantly associated with increased expression of 16
253 genes and decreased expression of five genes (hypoxic volume-associated gene signature,
254 HVS) (Figure 2A, Supplementary Table S3). Two genes overlapped with the 15-gene
255 hypoxia classifier, *LOX* and *PFKFB3*. On inspection, there was concordance between the
256 hypoxic volume-associated gene signature and expression of the 15-gene hypoxia classifier
257 (Figure 2A). The HVS was more highly correlated with each of the image features (hypoxic
258 volume, TMR and SUV_{max}) than the 15-gene hypoxia classifier, but only the hypoxic volume
259 was significantly correlated with HVS (hypoxic volume: rho=0.89, $P<10^{-4}$; TMR: rho=0.37,
260 $P=0.17$; SUV_{max}: rho=0.45, $P=0.09$; Spearman's correlation, two-tailed). Furthermore, the
261 HVS was significantly associated with CA9 IHC staining (Mann-Whitney U one-tailed,
262 threshold median of HVS score, $P=0.03$, Figure 2B).

263

264 To gain some initial insights into potential modes of gene regulation in tumours with a
265 ⁶⁴Cu-ATSM imaging hypoxic phenotype, miRNAs were ranked according to the association
266 of expression with increasing ⁶⁴Cu-ATSM hypoxic volume. The top-ranked genes for
267 association with the hypoxia volume signature were miR-21-5p ($p=0.004$, adjusted $p=0.99$)
268 and miR-10b-5p ($p=0.005$, adjusted $p=0.99$) (Figure 2A, Supplementary Table S4). The
269 study-derived HVS was then inspected in two publicly available data sets from head and
270 neck cancers with long-term follow up and derived from alternative gene expression
271 platforms; GEO series GSE686 (Chung *et al*, 2004), GSE65858 (Wichmann *et al*, 2015).
272 The genes comprising the HVS were concordantly expressed within each data set

(Supplementary Figure S6), which indicates that this signature may be widely relevant to head and neck tumour series. Finally, in exploratory analyses of each independent series, the HVS was found to be associated with poorer progression-free survival during a three year follow-up period (GSE65858; HR=1.5 [1.0-2.2], $P=0.047$) and there was a suggestive association with worse three-year recurrence-free survival (GSE686; HR=3.5 [0.8-16], $P=0.1$) (Supplementary Figure S6).

DISCUSSION

This study demonstrates that ^{64}Cu -ATSM defined hypoxic volume correlates with a previously reported hypoxia gene expression response, and may be an important imaging parameter to consider for assessment of hypoxia in oropharyngeal squamous cell carcinomas (OPSCCs) using PET. In addition, to describe the gene expression differences which underlie the PET imaging features, we identified a refined gene signature associated with ^{64}Cu -ATSM hypoxic volume in OPSCCs.

^{64}Cu -ATSM PET is well tolerated and demonstrates a spectrum of hypoxic imaging phenotypes in OPSCCs. Cu-ATSM has been investigated as a promising radiotracer for hypoxia imaging, which is a challenging area of research as hypoxia is spatially and temporally heterogeneous. Small clinical studies in HNSCC have reported that different imaging parameters correlate with progression or outcome, but there is currently no consensus, and the molecular mechanisms underlying these associations are not well understood. In addition, SUV_{max} , while widely reported, is a single voxel measure and may be sensitive to technical factors affecting PET (Adams *et al*, 2010). Minagawa *et al*. found that ^{62}Cu -ATSM SUV_{max} , but not TMR, was significantly different in 17 locally advanced head and neck cancer patients with and without residual or recurrent tumours (Minagawa *et al*, 2011). A study of 25 head and neck cancer patients indicated both increasing SUV_{max} and

TMR were associated with worse progression-free survival (Sato *et al*, 2014). Grassi *et al*. (Grassi *et al*, 2014) used ^{64}Cu -ATSM to define a biological target volume in 11 patients with HNSCC and found this parameter, along with SUV_{max} , to have high sensitivity but low specificity in predicting complete response to therapy. The same group reported that hypoxic tumour volume and hypoxic burden (hypoxic tumour volume x SUV_{mean}) in 18 patients with lung cancer or HNSCC were more robust prognostic parameters for progression free survival after a median follow-up of 14.6 months (Lopci *et al*, 2015), which is in agreement with our data suggesting that hypoxic volume is an informative feature in ^{64}Cu -ATSM PET scans. Our data suggest that hypoxic volume might be a more reliable correlate with gene expression patterns associated with hypoxia.

The optimal method for volume delineation is uncertain and requires further investigation. Grassi *et al*. (Grassi *et al*, 2014) used a cut off of 42% of the lesion SUV_{max} , but their subsequent study did not apply a fixed threshold for contouring (Lopci *et al*, 2015). The limited spatial resolution of PET makes defining the edge of tumour uptake difficult, but also means that PET imaging does not necessarily show the microregional, true heterogeneity of hypoxia within the tumour (Horsman *et al*, 2012). The mechanism of pO_2 dependence and factors that can affect cellular uptake and retention in both normoxic and hypoxic tissues is also unclear. Cu-ATSM may be an indirect marker for hypoxia, correlating with levels of the biological reductants NADH and NADPH (Yoshii *et al*, 2012). Hypoxia is one of a number of conditions leading to NADH accumulation, providing the cellular reduction potential which leads to tracer retention. Other factors such as multidrug resistance protein 1 (MDR1) expression (Liu *et al*, 2009) and CD133⁺ expression (Yoshii *et al*, 2012) have also been shown to influence Cu-ATSM uptake and retention, as well as cellular copper metabolism and processing (Hueting *et al*, 2014). Cu-ATSM is a promising radiotracer to detect hypoxia, but other possible sources of uptake need to be taken into consideration when interpreting the images and determining clinical utility.

We therefore inspected the genes comprising our proposed gene signature associated with hypoxic volume in OPSCCs. There were a number of upregulated genes relating to the development of hypoxia in tumours. For example, SOD2 is an antioxidant enzyme, which prevents redox-mediated damage of mitochondrial proteins, and is associated with aggressive cancers with enhanced cell migration and metastases. Stress such as hypoxia leads to increased reactive oxygen species (ROS) and tumours may increase their expression of SOD2 to prevent ROS-mediated DNA damage (Connor *et al*, 2007). Also among the upregulated genes, NAMPT tissue expression has also been found to be upregulated in tumours and shown to induce cell proliferation and angiogenesis (Shackelford *et al*, 2013). It is the rate-limiting enzyme for the biosynthesis of NAD essential for metabolism and energy production. Tumour cells have high metabolic rate and NAD consumption and therefore depend on the production of NAD, and hypoxia has been shown to result in NAMPT induction (Bae *et al*, 2006). NAMPT small molecule inhibitors are under investigation as a novel therapeutic, which reduce NAD levels resulting in ATP loss and inhibition of tumour cell proliferation (Xu *et al*, 2015). RUNX3 is downregulated in our samples with increasing hypoxic volume. RUNX3 has been shown to inhibit HIF-1 α stability through enhancing the interaction between HIF-1 α and PHD2, promoting HIF-1 α degradation in gastric cancer cells (Lee *et al*, 2014) with resulting inhibition of angiogenesis. Its expression has been shown to be downregulated in response to hypoxia and is frequently inactivated in gastric cancer, resulting in stimulation of proliferation and suppression of apoptosis (Lee *et al*, 2009). Supplementary Table S5 summarises the genes and their function.

CA9 is a transmembrane glycoprotein that is induced by hypoxia and considered an endogenous marker of hypoxia. It is a downstream target of hypoxia-inducible factor 1 (HIF-1) in the hypoxia response pathway and has been shown to be overexpressed in HNSCC due to hypoxia (Beasley *et al*, 2001). The association between the hypoxic volume and CA9, and between the 15-gene hypoxia classifier and CA9, further suggests that hypoxic volume

is an important feature for understanding hypoxic response in this series of OPSCCs, and that the gene expression from FFPE biopsy samples could reflect the hypoxia response of the tumour.

Among the miRNAs found to be associated with the hypoxic volume, miR-21 is frequently dysregulated miRNAs in HNSCC (Chang *et al*, 2008). Upregulation of miR-21 promotes cell proliferation, migration and inhibition of apoptosis, and is associated with poor prognosis (Lu *et al*, 2008). Mir-210, the hypoxia miRNA (Huang *et al*, 2010), had low base coverage in our analysis and was not associated with hypoxia expression signatures.

This study had several limitations. First, there was a small sample size which is typical of exploratory imaging-genomic studies with patients recruited from a single institution. However, a strong point of our study is that the PET studies were performed and analysed in a standardised way at a single PET centre. Second, the validation of our hypoxia-associated signature was limited to *in silico* functional investigations in the absence of a clinical validation set, and warrants further study to elucidate the mechanisms underlying these associations. Third, Cu-ATSM as a radiotracer provides hypoxic-to-normoxic contrast of sufficient quality to define a hypoxic volume but the optimal method for hypoxic volume delineation requires further investigation. Due to the existing clinical protocols for obtaining biopsy samples, it was not possible to attempt any registration (alignment) of biopsy sites and PET scans. Hypoxia responses detected in the diagnostic biopsy (CA9 hypoxia biomarker and gene expression profiling) were used to indicate the hypoxic status of the tumour, and more directly reflects clinical practise. These factors may be considered a limitation, and although outside the scope of this study, further work is needed to understand the effect of tumour heterogeneity on hypoxia evaluation in OPSCCs.

In conclusion, by combining PET imaging and mRNA expression profiling, our study revealed that Cu-ATSM PET hypoxic volume is associated with hypoxia gene signatures in

OPSCCs and suggests that PET could be a useful surrogate for hypoxia gene signatures in order to stratify patients for treatment. As with all exploratory biomarker studies, our findings now require prospective investigation in a larger number of patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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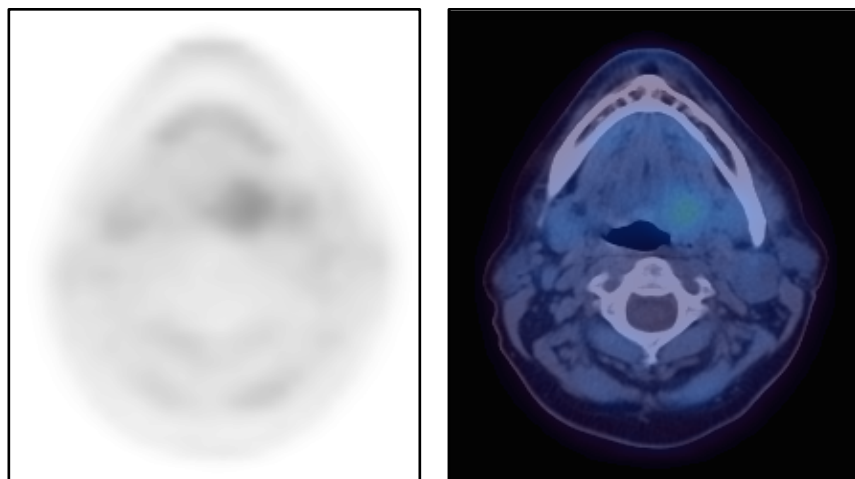
TITLES AND LEGENDS TO FIGURES

Figure 1. ^{64}Cu -ATSM PET imaging and association with CA9 immunohistochemical staining and a hypoxia gene expression response in diagnostic biopsies. A-B. Example images of ^{64}Cu -ATSM PET (left) and fused PET/CT (right) scans. (A) Patient with T2N2b left base of tongue SCC, with Cu-ATSM uptake in the primary but no uptake in left level II neck node. (B) Patient with T3N1 left tonsil SCC with high uptake in primary. **C.** Immunohistochemistry staining of hypoxia biomarker CA9 versus each image feature. 'High' is defined as values greater than the median value. **D.** Scatterplots of ^{64}Cu -ATSM imaging parameters versus the previously reported Sorensen2015 hypoxia classifier. Hypoxic volume, TMR and SUV_{max} plotted against the 15-gene hypoxia classifier. A significant positive correlation is observed between the hypoxia score and hypoxic volume, but not TMR or SUV_{max} . Red points indicate samples with low hypoxia score but high SUV_{max} . Samples from patients who had diagnostic tonsillectomies (Study IDs 9 and 11) were excluded as a hypoxic volume could not be determined.

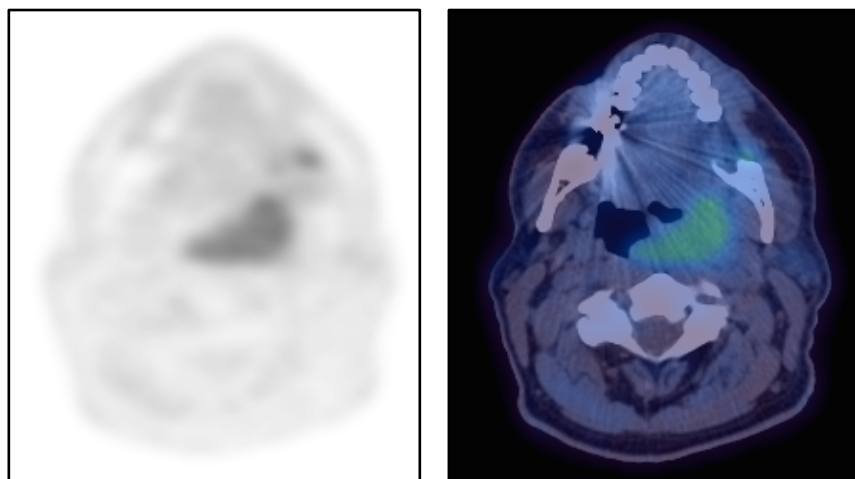
Figure 2. Hypoxia-associated gene signature (HVS) associated with hypoxic volume. **A.** The heatmap displays the HVS, comprising 16 genes positively and 5 genes negatively associated with hypoxic volume. Samples are ordered by increasing hypoxic volume (base mean > 200, adjusted $P < 0.05$; or *italicised*: adjusted $P < 0.1$). Two genes which overlapped with the 15-gene hypoxia classifier gene list are shown in bold. Yellow and blue colour bar shows the hypoxia 15-gene classifier score by rank within samples, for comparison (Sorensen2015). **B.** CA9 IHC is shown compared with 'low' or 'high' HVS signature scores for all samples ($n=15$). 'High' is defined as HVS scores greater than the median of HVS scores.

Table 1. Patient baseline characteristics and uptake values[illegible]

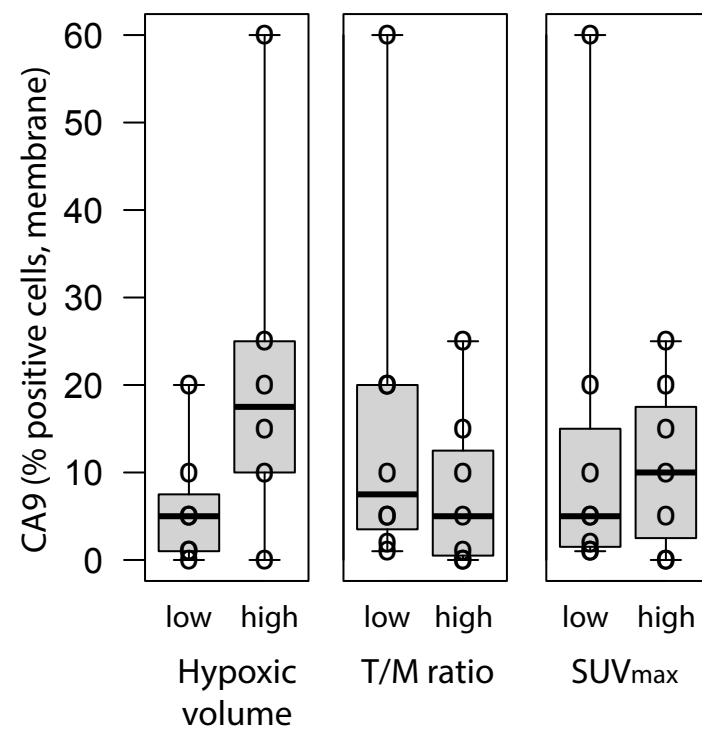
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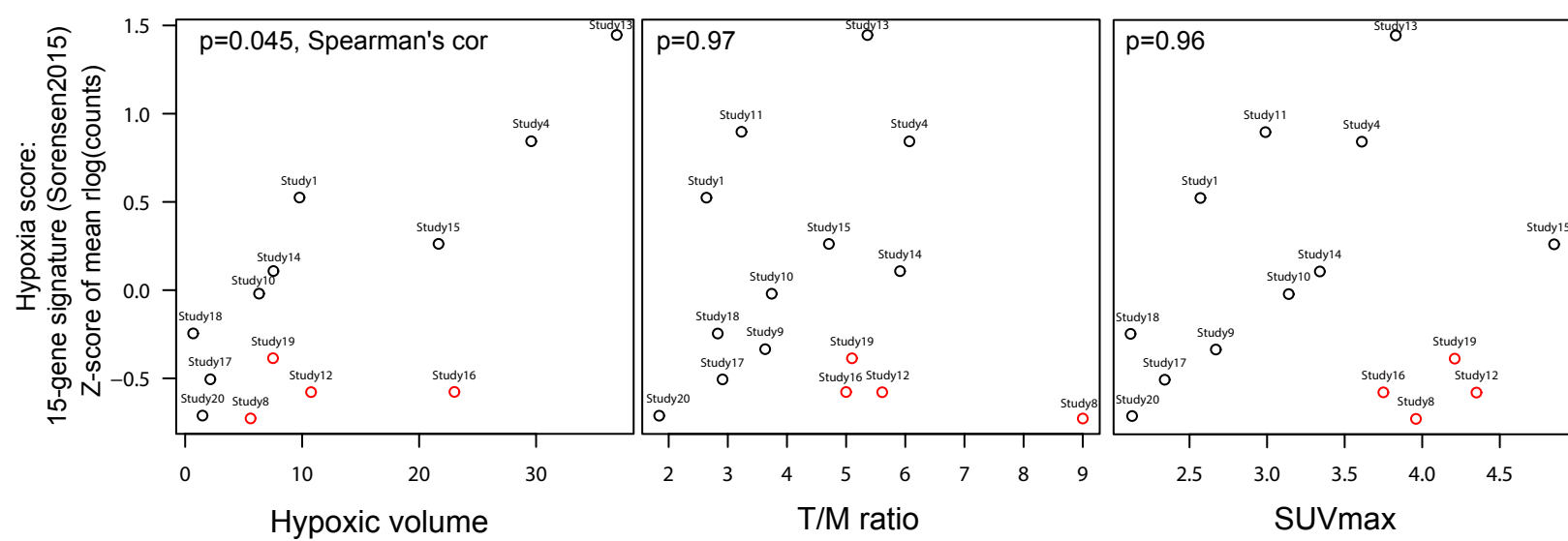
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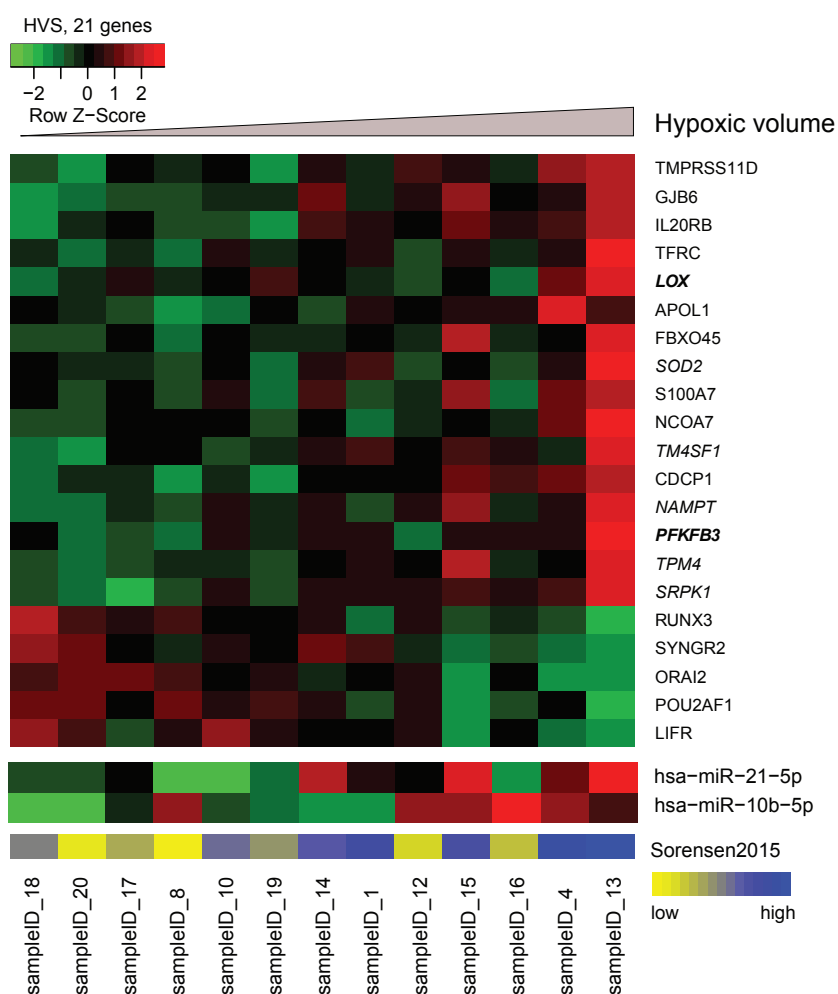
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